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Bacterial target-specific photocatalyst for the enhancement of antibacterial property to targets

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ABSTRACT

A bacterial target-specific titanium oxide (TiO_2) photocatalyst was developed for the enhancement of selective inactivation of targeted bacteria. An antibacterial composition comprising TiO_2 particles immobilized with a bacterial-specific antibody having affinity to bacteria of interest was prepared via a carbodiimide hydrochloride/N-hydroxysulfosuccinimide (EDC/NHS) coupling reaction between polyacrylic acid (PAA) coated TiO_2 and an antibody. As a model case, an antibody to *Escherichia coli* was conjugated with the PAA-coated TiO_2 (TiO_2-Ab_E). We evaluated the enhancement of the antibacterial effect of TiO_2-Ab_E against target *E. coli*, compared with its effect on other bacteria that lack affinity for the antibody used. The TiO_2-Ab_E inactivated approximately 90% of the *E. coli* at 15 min, whereas the raw TiO_2 inactivated approximately 20% of the *E. coli* after the same period of time under UV irradiation. The TiO_2-Ab_E did not show an enhanced antibacterial effect against non-target bacteria. We infer that close contact between TiO_2 particles and *E. coli*, as a result of the specificity of the antibody, can enhance the direct transfer of reactive oxygen species (ROS) generated by TiO_2 particles to the cellular surface under UV irradiation and result in rapid and efficient inactivation of the targeted bacteria. The strategy presented here will facilitate the combination of other receptors and TiO_2 particles for the preparation of highly selective and photocatalytic composites to prevent or remediate contamination by unwanted bacteria in a wide variety of natural and man-made systems.

1. Introduction

Human beings are frequently at risk of infection from a bacteria, fungi and viruses, through various routes of contact (nose, mouth, and skin) from the living environment. Infection by microorganisms can result in serious problems such as food poisoning, enteritis, and Severe Acute Respiratory Syndrome (SARS) [1–3]. Numerous antimicrobial methods, such as filtration [4,5], thermal treatment [6,7], antibiotic agents [8,9], disinfectants [10], and ultraviolet (UV) irradiation [11,12], have been studied and evaluated for their effectiveness against different microorganisms under varying environmental conditions. Interestingly, however, these studies have been mostly dedicated to the non-selective inactivation of microorganisms. Most methods for microorganism sterilization are non-specific, and therefore, useful microorganisms are killed alongside target microorganisms.

Titanium oxide (TiO_2), a photocatalyst, has been widely used in environmental fields due to its high chemical stability, excellent oxidation capability, good photocatalytic activity, low-cost of production, and non-toxicity [13,14]. In addition, TiO_2 photocatalysis has been intensively applied to the inactivation of a broad spectrum of microorganisms [15–17]. It has already been demonstrated that reactive oxygen species (ROS) such as hydroxyl radicals ($OH^{\bullet}$) and superoxide anions ($O_2^{\bullet -}$), produced by the photocatalytic activity of TiO_2 under UV irradiation, are reactive with microorganisms and can kill or deactivate bacteria, viruses, and cells more efficiently than UV alone [17–19]. Recently, this enhanced photocatalytic and antibacterial activity was demonstrated using TiO_2 particles loaded with metal oxides (Ag, Au, or Cu) or graphene under visible light [20–23]. However, previous studies of TiO_2 and modified TiO_2 photocatalysts showed no selectivity for target microorganisms where ROS were applied to such processes [12,17–25]. A small number of studies have been conducted on the bioconjugation of TiO_2 with DNA, biotin, or antibodies; however, these studies did not focus on the selective inactivation of bacterial targets [26–29].

In the present work, we prepared bacterial target-specific TiO_2 particles designed to enhance their antibacterial effect and to target and inactivate specific species of bacteria. We prepared TiO_2 particles conjugated with a bacteria-specific antibody (*Escherichia coli* polyclonal antibody, Ab_E) and evaluated whether the composite displayed enhanced selective antibacterial performance in

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targeting *E. coli*. The specificity of TiO_2-Ab_E was verified by measuring its effect on other bacteria not bound by the antibody, such as *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Citrobacter freundii* and *Bacillus subtilis*.

2. Materials and methods

2.1. Materials

TiO_2 (P-25, composition: 75% anatase and 25% rutile, surface area: 50 m^2/g) was purchased from the Degussa Company (Germany). Polyacrylic acid (PAA) was purchased from Wako Pure Chemical Industries, Ltd. (Japan). N,N-dimethylformamide (DMF), acetone, ethanol, ethanolamine, and 1-ethyl-3-[3-dimethylaminopropyl] were purchased from Sigma–Aldrich (St. Louis, MO USA). Carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), 2-(N-morpholine) ethanesulfonic acid (MES), sodium phosphate (PBS), and anti-*E. coli* polyclonal antibody (Rabbit IgG) were purchased from Thermo Fisher Scientific Inc. (Rockford, IL USA). Carboxylic acid Dynal M-270 magnetic beads were purchased from Invitrogen (Carlsbad, CA USA). Methylene blue was purchased from Samchun Pure Chemical (Korea). *E. coli* (KCTC 2571), *S. epidermidis* (KCTC 1917), *K. pneumoniae* (KCTC 2208), *E. aerogenes* (KCTC 2190), *C. freundii* (KCTC 2006), and *B. subtilis* (KCTC 1022) were purchased from the Korean Collection for Type Culture (KCTC). Nutrient broth and nutrient agar were purchased from Becton & Dickinson, Co. (Franklin Lakes, NJ, USA).

2.2. Preparation of PAA modified TiO_2 particles

TiO_2 particles (0.1 g) were dissolved in 20 ml of DMF solution and 2 ml of DMF containing 100 mg/ml of PAA was added, followed by mixing. The mixed solution was incubated for 5 h at 150 °C and then cooled to room temperature. Acetone (38 ml) was added to the cooled solution, which was then incubated for an additional 1 h at room temperature. The particles were recovered by centrifugation (4000 rpm) for 20 min at room temperature. The recovered PAA-coated TiO_2 particles (TiO_2-PAA) were washed with 20 ml of ethanol and then centrifuged (4000 rpm) for 20 min at room temperature. The TiO_2-PAA particles were dried for 24 h at room temperature. The dried TiO_2-PAA particles were solubilized using 2 ml of MES buffer (100 mM, pH 5.9) up to 0.05 g/ml for further usage. The surface of the TiO_2, TiO_2-PAA, and TiO_2-Ab_E was analyzed using an Infinity Gold Fourier transform infrared (FTIR) spectrometer (Thermo Mattson) at room temperature.

2.3. Antibody conjugation with TiO_2-PAA particle and magnetic beads

Suspended TiO_2-PAA particles (0.05 g/ml) were mixed with 1 ml of MES buffer (100 mM, pH 5.9) containing sulfo-NHS (20 mM) and EDC (80 mM) solution and then incubated for 1 h with shaking (200 rpm) at room temperature. The treated TiO_2-PAA particles were recovered by centrifugation (4000 rpm) for 20 min at room temperature and then re-suspended with 1 ml of MES buffer (100 mM, pH 5.9). The suspended TiO_2-PAA particles was mixed with 50 μl of antibody (4–5 mg/ml) and incubated overnight at 4 °C. Subsequently, the activated carboxyl residues were blocked using 0.5 ml of 0.1 M ethanolamine solution, and the mixture was incubated for 30 min at 4 °C. The antibody-conjugated TiO_2 (TiO_2-Ab_E) particles were recovered by centrifugation (4000 rpm) for 10 min at room temperature and then washed two times using 1 ml of PBS (pH 7.0) buffer. The washed TiO_2-Ab_E particles were re-suspended with 2 ml of PBS (pH 7.0) buffer up to 0.05 g/ml and stored at 4 °C until use.

For conjugation of the antibody with the magnetic beads, 20 μl of carboxylic acid magnetic beads (2×10^9 particles/ml, 30 mg/ml) were washed three times using MES buffer (25 mM, pH 5.0) and then mixed with 180 μl of MES buffer (25 mM, pH 5.0) containing sulfo-NHS (115 mM) and EDC (130 mM). The mixture was incubated for 1 h with shaking (250 rpm) at room temperature and washed three times using MES buffer (25 mM, pH 5.0). The washed magnetic beads were re-suspended using 200 μl of MES buffer (25 mM, pH 5.0) and mixed with 20 μl of *E. coli* polyclonal antibody (4–5 mg/ml). The mixture was incubated overnight at 4 °C after incubating for 1.5 h with shaking (200 rpm) at room temperature. Subsequently, the activated carboxyl groups were blocked with 180 μl of 0.1 M ethanolamine solution and the mixture was incubated for 30 min at room temperature. The antibody-conjugated magnetic beads were separated using magnet for 10 min at room temperature and washed three times using autoclaved deionized water. Finally, the antibody-conjugated magnetic beads were re-suspended with 80 μl of autoclaved deionized water up to 0.15 g/ml.

To confirm the binding of *E. coli* with TiO_2-Ab_E, the antibody-conjugated magnetic beads and TiO_2-Ab_E particles were mixed with or without *E. coli*. The *E. coli* culture (1×10^8 colony-forming units (CFU)/ml) was washed using PBS buffer (pH 7.0), re-suspended with 200 μl of PBS buffer (pH 7.0), and then incubated for 10 min at 37 °C with shaking (200 rpm) after mixing with 10 μl ($\sim 4 \times 10^6$ particles) of antibody-conjugated magnetic beads. Subsequently, 100 μl of TiO_2-Ab_E particles (1 mg/ml) were added and the mixture was incubated for 20 min with shaking (200 rpm) at 37 °C. The magnetic beads were washed three times using autoclaved water, and then separated using magnets. Finally, the mixture was dried for 24 h at room temperature. The surface morphology of particles was observed using a field emission scanning electron microscope (FE-SEM; S-4100; Hitachi).

2.4. Bacterial culture and investigation of antibacterial activity of particles

All bacteria used in this study were cultivated at 37 °C in nutrient broth medium (5.0 g of peptone, 3.0 g of beef extract in 1 L of distilled water) and all bacteria were cultured at a density of approximately 1×10^8 CFU/ml. Cultured bacteria were recovered by centrifugation (4000 rpm) for 20 min at room temperature and then washed two times using PBS buffer (pH 7.0) and used for the antibacterial activity test. During the antibacterial activity test, 100 μl of the bacterial samples were removed at each time point, diluted with autoclaved water, coated on a nutrient agar plate and incubated for 17 h at 37 °C to count CFUs. First, we tested the antibacterial activity of the TiO_2 and TiO_2-Ab_E particles. *E. coli* suspension (5 ml, 1×10^8 CFU/ml) was prepared in glass vials and mixed with 0.5 mg of raw TiO_2 or TiO_2-Ab_E particles. Before UV irradiation cell suspensions were incubated for 15 min in a shaking incubator (250 rpm) in order to allow the TiO_2 or TiO_2-Ab_E particles to bind with the *E. coli* before the UV lamp was turned on. A UV lamp (15 W, with a spectral maximum at 365 nm; Vilber Lourmat; France) was used to irradiate the cell suspensions in a shaking incubator (250 rpm) at room temperature. The distance between the UV lamp and the glass vials was 10 cm and the UV intensity was 0.7 W/cm^2. During the UV irradiation 100 μl of cell suspension was sampled to measure CFUs at each time point (0, 15, 45, 75, 115, and 135 min). To test for possible photodegradation and loss of photokilling activity of the TiO_2-Ab_E, particles were exposed under UV irradiation (15 W UV lamp, 365 nm, 0.7 W/cm^2) for 0 min, 30 min, 60 min or 120 min prior to use for photokilling experiment. Then, the photokilling activity of each particle type was tested against *E. coli*. UV irradiation was performed as previously described. CFUs were measured after 10 min of UV irradiation.

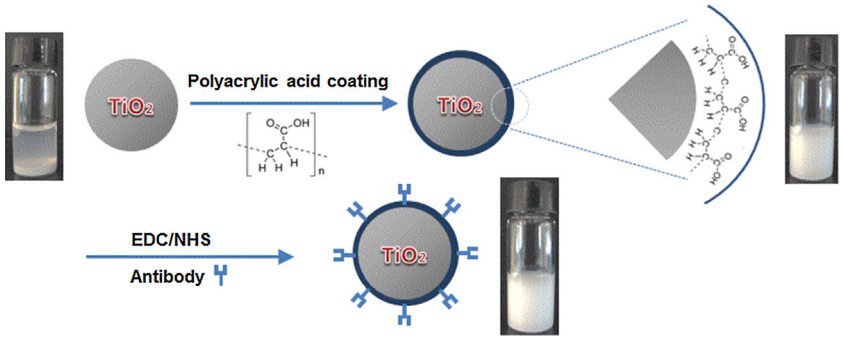

Fig. 1. Schematic illustration of the preparation of bacterial target-specific TiO_2 particles, where TiO_2 particles are surface-coated with polyacrylic acid (PAA), followed by conjugation of a polyclonal antibody via an EDC/NHS coupling reaction. The glass vials show the degree of suspension of TiO_2 particles in aqueous buffer at each step.

The effect of varying concentration of TiO_2 or TiO_2-Ab_E was also investigated. The *E. coli* suspension (2 ml, 1×10^8 CFU/ml) was transferred to glass vials and TiO_2 or TiO_2-Ab_E particles were added to the cell suspension at a final concentration of 0.01–0.5 mg/ml. The UV irradiation was performed as previously described, and the CFUs were measured after 20 min of UV irradiation.

The specificity of the photokilling effect of TiO_2-Ab_E was evaluated using *E. coli*, *S. epidermidis*, *K. pneumoniae* and *E. aerogenes*. For this experiment, we used a black box reactor containing a quartz vial, a magnetic stirrer and two 4 W UV lamps (max spectrum: 352 nm; Sankyo Denki; Japan) inside a box sealed to prevent entry of natural light. The UV intensity was 0.17 W/cm^2. The raw TiO_2 or TiO_2-Ab_E (final concentration of 0.1 mg/ml in mixture) was added to 20 ml of cell suspension (1×10^8 CFU/ml) in a quartz vial. Prior to UV irradiation, suspensions were magnetically stirred for 15 min in the dark, and stirring continued during UV irradiation. Aliquots of cell suspensions (100 μl) were moved from the quartz vial to measure CFUs at various time points during the UV irradiation process (0, 5, 20, 35, and 50 min). The specificity of the particles was also verified using two other species of bacteria, *C. freundii* and *B. subtilis*; CFUs of these two species were measured after 5 min of UV irradiation and compared with the previously tested bacteria.

The selective photokilling effect of TiO_2-Ab_E was tested using mixture of two species of bacteria, *E. coli* and *S. epidermidis*. Colony morphology differed so markedly between *E. coli* and *S. epidermidis* that their cell survival percentages could be easily measured using a colony counting method on nutrient agar plates. Before UV irradiation of the mixture, 15 min incubation was allowed for binding of TiO_2-Ab_E to its target bacteria. The cell survival percentage of each bacterial species was checked after 5 min of UV irradiation. Culture suspensions of *E. coli* (10 ml, 1×10^8 CFU/ml) and *S. epidermidis* (10 ml, 1×10^8 CFU/ml) were mixed in a quartz vial, and TiO_2 or TiO_2-Ab_E particles (final concentration of 0.1 mg/mL in mixture) were added. UV irradiation was administered in a black box reactor and CFUs were measured after 5 min. All experiments were repeated at least three times and average values with error bars are presented.

3. Results and discussion

3.1. Synthesis and characteristics of particles

Fig. 1 shows the steps required for preparation of the TiO_2-Ab_E particles. First, we modified the TiO_2 with a thin layer of PAA to address a carboxyl group, which can involve the conjugation of antibody with TiO_2 particles via EDC/NHS coupling reaction, and to prevent aggregation of TiO_2 in aqueous solution [30,31]. To confirm the coating of TiO_2 with PAA, raw TiO_2 and PAA-coated TiO_2 were suspended in deionized water. Raw TiO_2 particles were precipitated after a few minutes, whereas PAA-coated TiO_2 particles remained homogeneously suspended in water for several hours. These results indicate that the ionized carboxylic groups on the PAA-coated TiO_2 altered the surface characteristics of raw TiO_2 from hydrophobic to hydrophilic. Furthermore, FT-IR data confirmed the generation of carboxyl groups on the PAA-coated TiO_2 particles and the generation of an amide bond from the antibody attached to TiO_2-PAA (Fig. S1).

To explore the capability of TiO_2-Ab_E to specifically bind to *E. coli*, we used *E. coli* antibody-conjugated magnetic beads as *E. coli* capturing modules, and an additional sample of TiO_2-Ab_E was mixed with or without *E. coli*. We expected to observe aggregation of magnetic beads and TiO_2-Ab_E particles in the presence of *E. coli* because *E. coli* should act as a linker between antibody-conjugated magnetic beads and TiO_2-Ab_E particles. Fig. 2 shows the SEM images of the magnetic beads conjugated with *E. coli* antibody

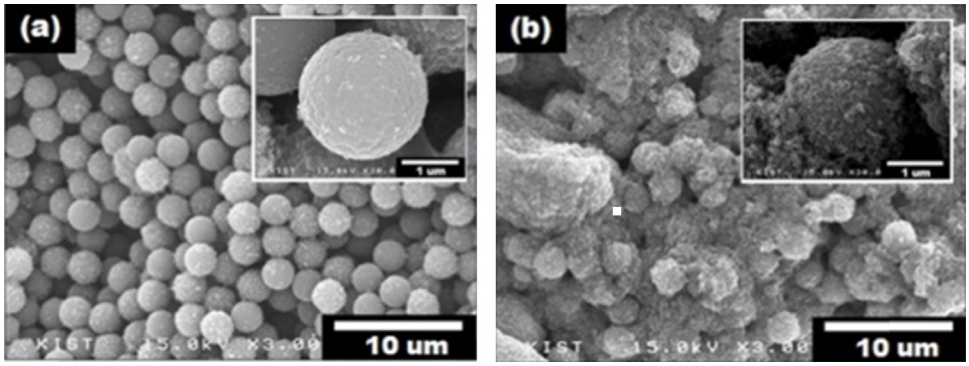

Fig. 2. Assay for TiO_2-Ab_E binding with or without *E. coli*. A magnetic bead conjugated with *E. coli* antibody was used as a cell capturing moiety and additional TiO_2-Ab_E was mixed (a) without *E. coli* or (b) with *E. coli*. Inset images have higher magnification. The scale bar (white line) of the inset images is 1 μm.

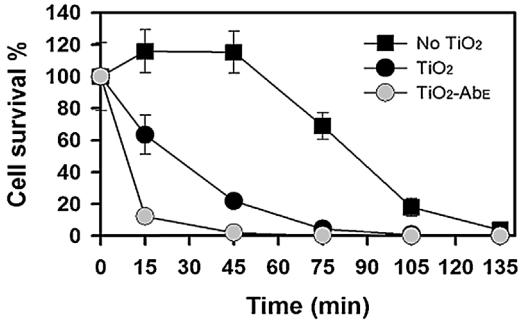

Fig. 3. Inactivation of *E. coli* by TiO_2 or TiO_2-Ab_E particles. Before UV irradiation the cell and TiO_2 or TiO_2-Ab_E mixture was incubated for 1 h. '0 min' denotes the starting point of UV irradiation.

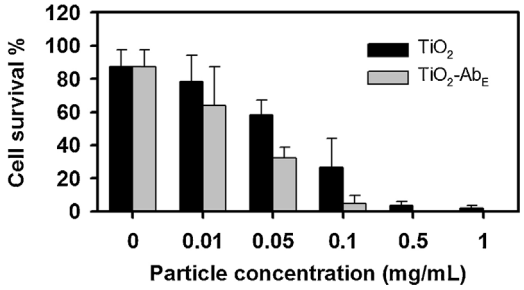

Fig. 4. Concentration dependency of antibacterial effect of TiO_2 or TiO_2-Ab_E on *E. coli* after 20 min of UV irradiation.

in the presence and absence of *E. coli*. No interaction was observed between the TiO_2-Ab_E particles and the magnetic beads in the absence of *E. coli*, whereas a sandwich complex of magnetic beads and TiO_2-Ab_E particles was observed when *E. coli* was present.

3.2. Inactivation of E. coli by raw TiO_2 or TiO_2-Ab_E particle

On the basis of the observed ability of TiO_2-Ab_E to bind to *E. coli*, we compared the photocatalytic activity of raw TiO_2 and TiO_2-Ab_E particles in terms of their antibacterial function. Fig. 3 shows the antibacterial effect of TiO_2 and TiO_2-Ab_E particles. TiO_2-Ab_E inactivated *E. coli* was much more strongly than raw TiO_2 particles. The TiO_2-Ab_E particles killed approximately 90% of *E. coli* at 15 min, whereas the raw TiO_2 particles killed 20% of the bacteria after the same period of time. To achieve a similar degree of cell inactivation (~95%), the TiO_2-Ab_E solution required 45 min, whereas the raw TiO_2 solution required 75 min, and the UV irradiation-only sample (no TiO_2) required 135 min. Thus, TiO_2-Ab_E has more rapid and more reliable antibacterial properties than raw TiO_2 particles or only UV irradiation alone. The antibacterial activity of target-specific TiO_2-Ab_E to *E. coli* was substantially enhanced compared to TiO_2 synthesized using the vapor condensation method or vanadium pentoxide-loaded TiO_2, which were synthesized for the enhanced catalytic activity of P25-TiO_2 and did not have bacterial target specificity [32,33].

Based on the results of the TiO_2-Ab_E and *E. coli* binding assay, we expect that close contact of TiO_2-Ab_E with *E. coli* (Fig. 2 and Fig. S2), via antibody-antigen interaction before or after UV irradiation, can enhance the antibacterial effect. TiO_2 photocatalysis inactivates microorganisms mainly by production of ROS and photons [19,34]. ROS usually damage external structures of microorganisms such as cellular outer membranes [35]. Accordingly, considering the short lifespan of ROS in aqueous solutions, we can expect that close surface contact of TiO_2 with *E. coli* would enhance the direct transfer of ROS, which is considered to be a major factor for rapid efficient killing of bacteria.

Furthermore the effect of the TiO_2 concentration was also investigated under 20 min UV exposure after addition of particles. For all concentrations, samples exposed to TiO_2-Ab_E showed lower cell survival percentages than those exposed to TiO_2 (Fig. 4).

It is possible that the PAA coating on the TiO_2 particles could block the release of ROS, and we therefore tested the photocatalytic activity of raw TiO_2 and TiO_2-PAA particles by comparing their photodegradation of methylene blue (MB) under UV irradiation. Raw TiO_2 particles degraded MB more strongly than TiO_2-PAA or TiO_2-Ab_E particles (Fig. S3). This result can be interpreted as indirect evidence that the PAA surrounding the TiO_2 particles prevented the release of ROS. Considering the possible reduction in the photocatalytic activity of TiO_2-PAA or TiO_2-Ab_E compared to raw TiO_2 particles indicated by the MB degradation experiment, the superior antibacterial performance of TiO_2-Ab_E can be explained by the close contact of the TiO_2 particles with the surface of target bacteria, in spite of the poor photodegradative performance of TiO_2-PAA (Fig. S2).

UV irradiation or ROS generation may also degrade the antibody on the surface of the TiO_2, which may decrease the antibacterial activity of TiO_2-Ab_E. The enhancement in TiO_2-Ab_E's photokilling activity decreased as UV exposure prior to use increased. The TiO_2-Ab_E particles exposed to UV for 120 min showed a 15% decrease in activity compared to non-exposed particles (Fig. S4). This may be due to loss of function of the antibody on the TiO_2 surface. Nevertheless, the functional activity and specificity to *E. coli* of TiO_2-Ab_E were not seriously diminished after even 120 min of UV exposure, and remained superior to raw TiO_2.

3.3. Specific photokilling effect of TiO_2-Ab_E

Next, we investigated the enhanced photokilling effect of TiO_2-Ab_E on the target bacterium. First, we investigated the antibacterial effect of TiO_2-Ab_E on *E. coli*, as well as *S. epidermidis*, *K. pneumoniae* and *E. aerogenes*, which have no affinity with the *E. coli* specific antibody used. As shown in Fig. 5, no obvious changes were observed in cell survival percentages of *S. epidermidis*, *K. pneumoniae*, or *E. aerogenes* when raw TiO_2 or TiO_2-Ab_E was mixed with each culture

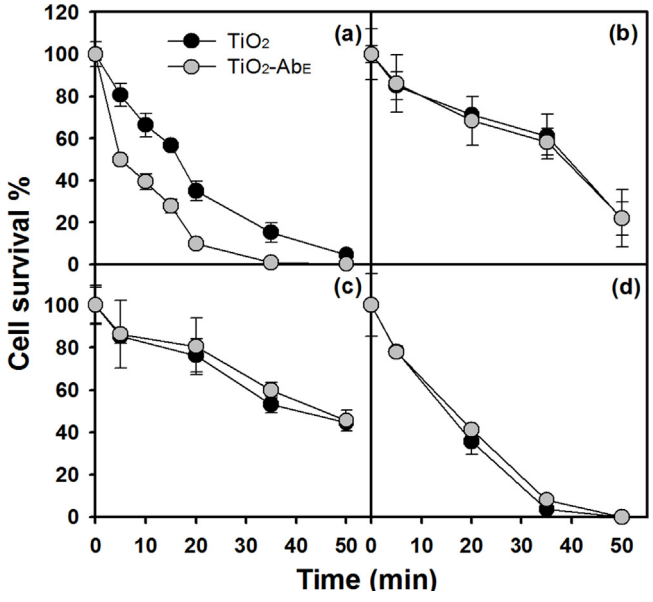

Fig. 5. A comparison of cell survival ratios of (a) *E. coli*, (b) *S. epidermidis*, (c) *K. pneumoniae* and (d) *E. aerogenes* as a function of the UV illumination time in the presence of TiO_2-Ab_E or TiO_2 particles.

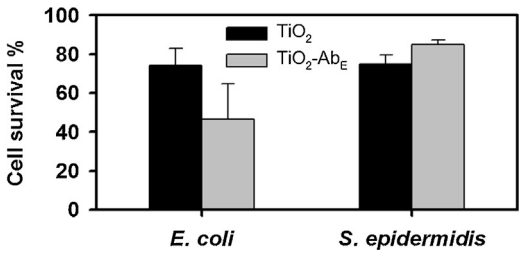

Fig. 6. The antibacterial effect of TiO_2-Ab_E in a mixed culture of *E. coli* and *S. epidermidis* after 5 min of UV irradiation.

under UV irradiation. Only *E. coli* showed a difference in cell survival percentage, and TiO_2-Ab_E killed *E. coli* more rapidly than raw TiO_2 particles. We compared the cell survival ratio of each species of bacteria after 5 min UV irradiation, including two additional bacteria, *C. freundii* and *B. subtilis*, with TiO_2 or TiO_2-Ab_E. Only *E. coli* showed a relatively decreased cell survival percentage in the presence of TiO_2-Ab_E compared to raw TiO_2 particles (approximately 30% difference) after 5 min of UV treatment, while the other five species of bacteria showed no significant difference in cell survival percentage (Fig. S5).

We further tested the enhanced photokilling effect of TiO_2-Ab_E under conditions of bacterial co-existence, using *E. coli* and *S. epidermidis*. We mixed cultures of *E. coli* and *S. epidermidis*, and then added raw TiO_2 or TiO_2-Ab_E particles to the bacterial mixture. As shown in Fig. 6, addition of TiO_2-Ab_E resulted in a 55% reduction in the cell survival percentage of *E. coli* whereas addition of raw TiO_2 resulted in a 30% reduction. The cell survival ratio of *S. epidermidis* did not show any difference after 5 min of UV irradiation with either raw TiO_2 or TiO_2-Ab_E. The enhanced inactivation of target bacteria in a mixed population implied that TiO_2-Ab_E binds specifically to its target, *E. coli*, in a mixed bacterial population, with little non-specific binding to non-target bacteria. Thus, accumulation of TiO_2 particles around the target cell surface enhanced the inactivation of target bacterial cells in a mixed population.

4. Conclusions

We prepared TiO_2 particles conjugated with a bacterial antibody and measured the enhancement of their bacteria-specific photokilling activity under short periods of UV exposure. In the presence of UV, TiO_2-Ab_E enhanced the photokilling of *E. coli* specifically and had no significant photokilling effect on non-target. We suspected that the close contact of TiO_2 particles with bacterial cells, due to the high binding affinity of the conjugated receptors to their targets, may enhance ROS transfer to the bacterial surface, resulting in the enhancement of photokilling activity, although the oxidation ability of PAA-coated TiO_2 was weaker than that of raw TiO_2. TiO_2-Ab_E also inactivated *E. coli* more efficiently than non-target bacteria in a mixed population. The strategy presented in this study will facilitate the combination of other receptors with TiO_2 particles for preparation of highly selective and photocatalytic composites or antibacterial coating materials to remediate a wide variety of natural and man-made systems from contamination by unwanted organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apcatb.2013.11.038.

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